

## Association between *GSTM1*\*0 and squamous dysplasia of the esophagus in the high risk region of Linxian, China

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Received 9 February 2000; accepted 30 March 2000

### Abstract

Individuals with specific phase I and phase II enzyme polymorphisms may be at increased risk for squamous cell carcinoma of the esophagus. However, to our knowledge there has been only one previous report that evaluates a potential role for these polymorphisms in increasing risk for preneoplastic squamous lesions of the esophagus. To explore this further, we examined polymorphisms in *CYP1A1*, *CYP2E1*, *GSTM1* and *GSTT1*, both independently and in combination, for potential associations with the risk of biopsy-proven squamous dysplasia of the esophagus in asymptomatic adults from Linxian, a high risk region in China. Cases consisted of 56 individuals from an esophageal cancer screening study with an endoscopic biopsy diagnosis of mild or moderate squamous dysplasia. Each case was matched on age ( $\pm 1$  year) and gender to a control. Controls were defined as screening study participants with an endoscopic biopsy diagnosis of normal mucosa or esophagitis. DNA was extracted from frozen cell samples obtained by cytologic balloon examination and genotyped using standard methods. Individuals who were *GSTM1* null (homozygous for *GSTM1*\*0) were found to have a tendency for an increased risk of esophageal squamous dysplasia (odds ratio = 2.6, 95% CI, 0.9–7.4). No excess risks were observed for inheritance of other putative at risk genotypes *CYP1A1*\*2B, *CYP2E1*\*6 or *GSTT1*\*0. The risk associated with the inheritance of combined genotypes was not significantly different than the risk estimates from the univariate analysis. These results are consistent with the notion that exposure to environmental carcinogens that are detoxified by *GSTM1*, such as polycyclic aromatic hydrocarbons, may contribute to the etiology of esophageal cancer in Linxian. Published by Elsevier Science Ireland Ltd.

**Keywords:** Esophageal cancer; Cytochrome P450; Glutathione-S-transferase; China

### 1. Introduction

Linxian, China has some of the highest incidence rates of esophageal squamous cell carcinoma (ESCC)

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in the world [1]. Although the etiology for these high rates remains unknown, studies suggest that they may be associated with exposure to environmental carcinogens, including polycyclic aromatic hydrocarbons and nitrosamines [2–6]. The biologic consequences of carcinogenic environmental exposures may be modulated by polymorphisms in carcinogen metabolism and detoxification genes, which may result in differences in esophageal cancer risk among individuals with similar carcinogen exposures. Phase I enzymes metabolically activate parent compounds and procarcinogens to reactive species that exert their tumorigenic potential through binding to cellular macromolecules. These Phase I enzymes include cytochrome P450s such as *CYP1A1* and *CYP2E1* [7]. Aryl hydrocarbon hydroxylase, or *CYP1A1*, is believed to be responsible for the metabolic activation of benzo(a)pyrene and other polycyclic aromatic hydrocarbons. Two recent Japanese studies found no association between *CYP1A1* polymorphisms and ESCC [8,9], while a third found an increased frequency of the at risk *CYP1A1*\*2B genotype in ESCC cases [10]. *CYP2E1* is believed to be responsible for the metabolic activation of various *N*-nitrosamines, and contains both RsaI (*CYP2E1*\*5B) and DraI (*CYP2E1*\*6) polymorphisms. Studies correlating the *CYP2E1*\*5B genotype to ESCC risk have reported inconsistent results. A study in a Japanese population found no difference in allele frequencies between ESCC cases and controls [9], but another study from Linxian found a significantly increased risk of developing ESCC or esophageal adenocarcinoma among persons with a *CYP2E1*\*5B genotype [11]. The only study to date examining the *CYP2E1*\*6 polymorphism found no association with ESCC or esophageal adenocarcinoma risk [11].

In addition to activation, carcinogens may be detoxified by Phase II enzymes such as the glutathione-S-transferases which include mu- (*GSTM1*) and theta- (*GSTT1*) isoforms. *GSTM1* detoxifies the reactive metabolites of benzo(a)pyrene and other polycyclic aromatic hydrocarbons [12]. A homozygous deletion in the coding region of *GSTM1* has been associated with an increased risk of breast cancer and squamous cell carcinoma of the lung [13,14]. Also, heavy smokers with combined *CYP1A1* valine homozygous (*CYP1A1*\*2B) and *GSTM1* null genotypes have been found to be at a statistically higher risk for esophageal cancer than

control subjects [10]. *GSTT1* detoxifies smaller reactive compounds, such as ethylene oxide and diepoxybutane [15]. A homozygous coding region deletion results in a deficient genotype that has been associated with gastric cancer [16], an early age of onset of colon cancer [17], and with the myelodysplastic syndrome [18]. In addition, compared with healthy controls, a combination of *GSTM1* and *GSTT1* null genotypes has been found in higher frequency in patients with squamous cell carcinomas of the head and neck, or lung, cancer types that are histopathologically and etiologically similar to squamous cell carcinoma of the esophagus [19,20].

Thus, evidence suggests that an exposed individual with specific Phase I and Phase II enzyme genotypes may have an increased risk of developing squamous esophageal carcinoma. It is yet unknown if an association exists between specific genotypes of these xenobiotic enzymes and preneoplastic squamous lesions of the esophagus. Understanding these early relationships may help us to better explain the gene-environment interactions associated with esophageal cancer initiation and promotion. These interactions may be especially important in high risk regions such as Linxian.

Previous studies from our group have shown that squamous dysplasia is the near-term histologic precursor of esophageal squamous carcinoma in Linxian. This is true both for moderate and severe dysplasia [21] and for mild dysplasia (Dawsey, unpublished data). We have also shown that endoscopic biopsy sampling directed by mucosal iodine staining is an accurate way of distinguishing patients with and without esophageal squamous dysplasia [22]. The current study examines whether certain genetic variants of *CYP1A1*, *CYP2E1*, *GSTM1*, and *GSTT1*, independently or in combination, are associated with increased risk of biopsy-proven squamous dysplasia of the esophagus in asymptomatic adults in Linxian.

## 2. Materials and methods

The general design of this study was to analyze frozen cell samples collected as part of an esophageal cytologic screening study (described in greater detail in Roth et. al. [23] for associations between *CYP1A1*, *CYP2E1*, *GSTM1* and *GSTT1* genotypes and the

presence of biopsy proven squamous dysplasia in asymptomatic individuals. This study was approved by the Institutional Review Boards of the collaborating institutions, the Cancer Institute of the Chinese Academy of Medical Sciences (CICAMS), and the National Cancer Institute and the National Institute for Occupational Safety and Health in the United States.

### 2.1. Patient population

Participants were recruited in the spring of 1995 from 11 villages in Linqi Commune, an administrative district in the south of Linxian. Although Linxian is well-known for its high rates of esophageal cancer, and Northern Linxian has been the site of many previous studies, the inhabitants of Linqi Commune had not previously been studied in a systematic manner by cytologic or endoscopic techniques. All 50–69 year old residents in the 11 villages were asked to participate, unless they had a history of cirrhosis, esophageal varices, vomiting blood, or a reaction to topical anesthetics or iodine, or were considered too weak to undergo the examinations.

### 2.2. Cytologic balloon examinations

The details of the procedures and methods used for the cytologic balloon and endoscopic examinations have been previously reported [23]. Briefly, the balloon sampler (Fifth Rubber Manufacturing Co., Shanghai, China) was inserted by a technician into the back of the throat and the patient was asked to swallow. Once in the stomach, the balloon was inflated with 20–30 cm<sup>3</sup> of air, and then gradually pulled back up the esophagus. When the balloon reached 18 cm from the incisor teeth, all the air was released and the balloon was completely withdrawn. After removal, the balloon was smeared directly onto four slides for cytologic examination. The cells and mucoid debris that remained adherent to the deflated balloon sampler were suspended in saline and then centrifuged at 1500 rev./min for 5 min. The pellet was then resuspended in a few drops of supernatant, transferred to a 1.8 cm<sup>3</sup> screw top tube (Nalge Nunc, Roskilde, Denmark), flash frozen in liquid nitrogen, and then held at –80°C until subsequent analysis.

### 2.3. Endoscopic examinations

The patients underwent endoscopy in May 1995, approximately 1 month after their cytology exams. The examinations were done before the cytology slides were read, so the endoscopist was unaware of the cellular adequacy or diagnosis of the cytology smears.

Endoscopy was performed by one of the authors (G.Q.W) using an Olympus GIF-130 videoendoscope (Olympus Corporation, Tokyo, Japan). After insertion of the endoscope, the entire esophagus and stomach were examined, and all visible lesions were described and photographed. Then 20–30 ml of 1.2% Lugol's iodine solution (12 g I + 24 g KI in 1000 ml water) was sprayed from the gastroesophageal junction to the upper esophageal sphincter using a spray catheter passed through the biopsy channel. Lugol's iodine stains normal squamous mucosa brown, but leaves foci of squamous dysplasia and carcinoma and all glandular mucosa unstained. One or more 2.8 mm biopsies were taken from each unstained area and from at least one normally stained mid-esophageal site.

### 2.4. Biopsy slide reading

The endoscopic biopsy slides were read by one of the authors (M.J.R. or S.M.D.), using criteria previously described [21,24]. Each patient was classified according to their worst overall squamous endoscopic biopsy diagnosis.

### 2.5. Preparation of human DNA samples

The frozen cells obtained during esophageal cytologic sampling were pulverized in liquid nitrogen and stored at –80°C. High molecular weight DNA was prepared using the Promega Kit (Promega Corp., Madison, WI) with the inclusion of RNase (50 µg/ml in 0.6% sodium dodecyl sulfate, 10 mM EDTA, 10 mM Tris–HCl) and proteinase K (200 µg/ml) incubations at 37°C for 1 h. The DNA was precipitated with ethanol (2.5 volumes) and NaCl (100 mM), dried and dissolved in water (100 µl). Samples were quantified by UV absorbance spectrophotometry and DNA purity determined by A<sub>260</sub>/A<sub>280</sub>. The yield of DNA was less than 10 µg, with some samples less than 1 µg, and A<sub>260</sub>/A<sub>280</sub> ratios were typically 1.7–1.9.

## 2.6. Genotyping

Genotypes for *CYP1A1*\*2B, *CYP2E1*\*6, *GSTM1*\*0 and *GSTT1*\*0 were determined using modified standard PCR or PCR–RFLP methods [25,26]. Briefly, constitutive DNA samples, prepared from residual materials obtained following balloon cytological examinations, were melted in standard buffers ( $\text{MgCl}_2$  [2–3mM], dNTP [12mM], Taq Buffer (Perkin–Elmer, Branchburg, NJ and Foster City, CA); 94°C, 5 min) containing Amplitaq Gold™ or Amplitaq DNA™ (1 unit, Perkin–Elmer, Branchburg, NJ and Foster City, CA) and gene specific primers (*CYP1A1* – 5′-CCACTTCAGCTGTCTCCCTC-3′ and 5′-GCCAGGAAGAGAAAGACCTCCCAGC-GGTCAA-3′; *CYP2E1* – 5′-ATCATGTTGCCAG-GAAGG-3′ and 5′-GTCAGACCCTGGCTTTTCTT-3′; *GSTM1* – 5′-CTGCCCTACTTGATTGATGGG-3′ and 5′-CTGGATTGTAGCAGATCATGC-3′; *GSTT1* – 5′-TTCCTTACTGGTCTCTCATCTC-3′ and 5′-TCACCGGATCATGGCCAGCA-3′). Polymerization was allowed to proceed using a Perkin–Elmer 9600 thermocycler (*CYP1A1* and *GSTT1* – 94°C for 30 s, 56°C for 60 s, 72°C for 60 s, for 30 cycles, extension 6 min at 72°C; *CYP2E1* – 94°C for 60 s, 58°C for 60 s, 72°C for 60 s, for 35 cycles, extension 10 min at 72°C; *GSTM1* – 95°C for 60 s, 60°C for 60 s, 72°C for 60 s, for 35 cycles, extension 10 min at 72°C). Amplicons were subjected to analysis on agarose gels (2–3%) either before (*GSTM1*/*GSTT1*) or after restriction digestion (*CYP1A1* [HincII]/*CYP2E1* [DraI], restriction enzymes were obtained from New England Biolabs, Beverly, MA). Amplicons or restriction fragments were visualized using ethidium bromide (*CYP1A1* – 148/118/41/31bp; *CYP2E1* – 339/225/114bp; *GSTM1* – 170bp; *GSTT1* – 480bp; where *CYP1A1* multiplexed with *GSTT1* served as a positive control for *GSTT1* null and an amplicon of actin served as a positive control for *GSTM1* null).

## 2.7. Analysis

Cases consisted of 56 individuals identified from an esophageal cancer cytology screening study with a worst endoscopic biopsy diagnosis of mild ( $n = 23$ ) or moderate ( $n = 33$ ) squamous dysplasia. Each case was matched on age ( $\pm 1$  year) and gender to one

control, defined as screening study participants with an endoscopic biopsy diagnosis of normal mucosa ( $n = 53$ ) or esophagitis ( $n = 3$ ). One case of dysplasia could not be age and gender matched with a control and, therefore, was matched with an age appropriate member of the opposite sex. The frozen cell samples from individuals who had a worst endoscopic biopsy diagnosis of severe squamous dysplasia or cancer had been entirely used as part of another study and, therefore, could not be included. Samples were identified in the laboratory by a number (1–112) that gave no indication of case-control status.

## 2.8. Statistical analysis

The Chi-square and Fisher's exact test for heterogeneity was used to test the hypothesis that the distribution of allele prevalence was the same for cases and controls. Multivariate logistic regression techniques were used to examine the data for a potential association between genotypes and squamous dysplasia of the esophagus. All regression models were adjusted for age and gender. All analyses were performed using the statistical software package STATA (STATA Corporation, College Station, TX).

## 3. Results

The 112 participants had a mean age (SD) of 60.7 (6.2) years. Histologically, the controls consisted almost entirely of subjects with a worst overall squamous endoscopic biopsy diagnosis of normal and the cases consisted predominantly of subjects with a worst overall squamous endoscopic biopsy diagnosis of moderate dysplasia. Due to the limited amount of DNA obtained from some of the frozen cell samples, not all markers could be analyzed for all specimens. The number of successes as a percentage of total attempts were 93% for *CYP1A1*, 56% for *CYP2E1*, 70% for *GSTM1*, and 94% for *GSTT1*. However, all of the subjects were analyzed for at least one polymorphism and many of the samples ( $n = 51$  (46%)) were successfully amplified for all four of the polymorphisms included in the study. There were no significant differences in the number of successful and unsuccessful amplifications by case-control status for any of the genetic polymorphisms ( $P \leq 0.22$  for all comparisons).

Table 1  
Summary of allelic frequency by histologic subtype<sup>a</sup>

	<i>CYP1A1</i> (n = 104) <sup>b</sup>				<i>CYP2E1</i> (n = 63) <sup>b</sup>				<i>GSTT1</i> (n = 105)			<i>GSTM1</i> (n = 78)		
	MM	Mm	mm	FM	MM	Mm	Mm	FM	Functional (+)	Null (–)	F-Null <sup>c</sup>	Functional (+)	Null (–)	F-Null
Dysplasia	29	21	4	0.73	4	25	4	0.50	20	34	0.63	7	34	0.83
Control	29	17	4	0.75	3	22	5	0.47	19	32	0.63	13	24	0.64
Total	58	38	8	0.74	7	47	9	0.48	39	66	0.63	20	58	0.74

<sup>a</sup> MM, homozygous major variant; Mm, heterozygote; mm, homozygous minor variant; FM, frequency of major allele.

<sup>b</sup> Hardy–Weinberg equilibrium *CYP1A1*  $P = 0.41$ ; *CYP2E1*  $P = 0.00004$ .

<sup>c</sup> F-Null, frequency of the null genotype.

Table 1 shows the allelic frequencies of the four enzyme polymorphisms, by histologic subtype. The genotypic distribution for the *CYP1A1*\*2B polymorphism was found to be consistent with Hardy–Weinberg equilibrium (Table 1); and the frequency of the major allele (Ile: 0.74) was similar to that previously reported by others for Chinese populations (0.80) [27]. For *CYP2E1* a different pattern was seen and the genotypic distribution for the *CYP2E1*\*6 polymorphism was found to deviate significantly from the expected Hardy–Weinberg proportions. The data indicate an excess of heterozygotes and reductions in both categories of homozygotes. The frequency of the expected minor variant (0.52) was also found to be higher than previously reported for Asian populations (0.24–0.26) [27,28]. These differences were not driven by association with disease status.

Population frequency for the *GSTT1*\*0 genotype in both cases and controls (0.63) was similar to those previously reported for Chinese and Asian populations (0.60–0.64) [27–29]. This is higher than for populations of both African and European origin [29]. For *GSTM1*\*0, however, the homozygous *GSTM1*\*0 genotype was more frequent than expected (0.74 compared with previous reports in the range 0.56–0.63) [29,30] and this difference appeared to be driven by disease status. The frequency of the *GSTM1* null genotype among controls was similar to previous reports (0.64), but among cases it was higher (0.83). Thus, the *GSTM1* null genotype appeared to be associated with development of esophageal dysplasia (OR = 2.63; 95%CI = 0.93–7.39;  $P = 0.07$ ) (Table 2). No significant differences between the cases and controls were found after pooling the *CYP1A1* or *CYP2E1* heterozygotes with

their homozygous minor variants and comparing the overall frequencies of these genotypes to their major variant-homozygotes (Fisher's exact  $P > 0.69$  for both *CYP1A1* and *CYP2E1*) (Table 2).

Cases and controls were cross classified according to *CYP1A1*, *GSTM1* and *GSTT1* genotypes to look for possible biological relationships and functional interdependencies among these genotypes. *CYP2E1* was excluded because so few of the participants were homozygous for the major allele. The results of the

Table 2  
Estimated risk of squamous dysplasia by genotype<sup>a</sup>

Genotype <sup>b</sup>	Dysplasia	Control	OR (95% CI)
<i>CYP1A1</i> (MM)	29	29	1.00 (ref)
<i>CYP1A1</i> (Mm and mm)	25	21	1.19 (0.55–2.57) N.S.
<i>CYP2E1</i> (MM)	4	3	1.00 (ref)
<i>CYP2E1</i> (Mm and mm)	29	27	0.80 (0.18–3.56) N.S.
<i>GSTT1</i> (functional)	20	19	1.00 (ref)
<i>GSTT1</i> (Null)	34	32	1.01 (0.46–2.21) N.S.
<i>GSTM1</i> (functional)	7	13	1.00 (ref)
<i>GSTM1</i> (Null)	34	24	2.63 (0.93–7.39) $\chi^2 = 3.33$ ( $P = 0.07^c$ )

<sup>a</sup> Estimates controlled for baseline matching criteria (age and gender).

<sup>b</sup> MM, homozygous major variant; Mm, heterozygote; mm, homozygous minor variant.

<sup>c</sup> Pearson  $\chi^2$  test for homogeneity.

combined genotype analysis were similar to the univariate risk estimates (Table 3).

#### 4. Discussion

Linxian, China has epidemic rates of squamous esophageal cancer, the causes of which remain to be identified. Many previous studies in Linxian have focused on environmental risk factors, and a few have investigated genetic risk factors, but only recently have studies begun to evaluate the possible significance of gene-environment interactions. One potentially important gene-environment interaction is the effect that genetic polymorphisms of carcinogen metabolizing enzymes may have on the cancer risk of individuals with similar carcinogen exposure. To date, only four studies have compared genetic polymorphisms of xenobiotic enzymes with risk of developing ESCC, three in Japanese populations [8–10] and one in Linxian [11]. The current study is the first to correlate genetic polymorphisms of these enzymes with the presence of biopsy-proven esophageal squamous dysplasia, the near-term histologic precursor of ESCC in Linxian.

The strengths of the current study include classification of patients by endoscopic biopsies directed by mucosal iodine staining [22] and read using histologic criteria that have been shown to identify precursor lesions of ESCC in Linxian [21]. Limitations of this study include the relatively small sample size (especially important during the analysis of combined

genotypes) and the absence of cases with biopsy-proven severe squamous dysplasia.

The most striking finding in our study was a 2.6 times increase in risk of having esophageal squamous dysplasia among patients with the *GSTM1\*0* genotype, a result which approached statistical significance (95% CI. 0.93–7.39,  $P = 0.07$ ) even with our relatively modest sample size. This is a stronger association than has been seen in the previous reports comparing the *GSTM1\*0* genotype with ESCC risk [8–11], but it is consistent with other studies that associate this genotype with cancer development in other organs [13,14]. This association of the *GSTM1\*0* genotype with squamous dysplasia risk implies an early effect in the development of ESCC and is consistent with polycyclic aromatic hydrocarbons (PAHs) being one etiologic possibility contributing to the high rates of ESCC in Linxian.

We found no association between the at risk variants of *CYP1A1\*2B*, *CYP2E1\*6*, or *GSTT1\*0* genotypes, alone or after cross-classification, and the presence of esophageal squamous dysplasia. These findings are consistent with all but one of the previous reports relating genetic polymorphisms of these enzymes and ESCC risk [8–11]. In that study, Nimura et al. [10] found an increased frequency of the *CYP1A1\*2B* genotype in ESCC cases and an increased risk of ESCC among individuals with the combination of *CYP1A1\*2B* and *GSTM1\*0* genotypes. Differences between our study and this previous report include the populations studied and the stages of carcinogenesis evaluated.

Table 3  
Estimates of combined genotype on risk of esophageal squamous dysplasia<sup>a</sup>

		<i>CYP1A1</i>		<i>GSTT1</i>	
		MM	Mm + mm	Functional	Null
<i>GSTM1</i>	Functional	1.00 ( $n = 7$ )	0.33 (0.05–2.26) ( $n = 12$ )		
	Null	1.11 (0.23–5.38) ( $n = 31$ )	3.07 (0.57–16.46) ( $n = 24$ )		
<i>GSTT1</i>	Functional	1.00 ( $n = 28$ )	1.20 (0.30–4.86) ( $n = 11$ )		
	Null	1.00 (0.36–2.80) ( $n = 30$ )	1.19 (0.44–3.21) ( $n = 35$ )		
<i>GSTM1</i>	Functional			1.00 ( $n = 7$ )	0.67 (0.10–4.59) ( $n = 13$ )
	Null			1.48 (0.26–8.57) ( $n = 20$ )	3.14 (0.57–17.10) ( $n = 36$ )

<sup>a</sup> MM, homozygous major variant; Mm, heterozygote; mm, homozygous minor variant.

It is also important to mention differences between our study and the one previous study of genetic polymorphisms conducted in Linxian [11]. That study combined cases diagnosed by histology and by cytology, although these methods have not been shown to correlate well [31], it combined cases of precursor lesions and cancers, and it combined cases of squamous and glandular neoplasia, which probably have different biologic progressions and may well have different etiologic risk factors. It is not possible from the data presented in the previous study to find the genotype prevalences or risks associated with histologically confirmed squamous precursor lesions, which are the data that would be comparable to the results of the present study.

Inasmuch as an association was observed between *GSTM1* null individuals and esophageal dysplasia, these findings are consistent with our understanding of the biologic activity of these polymorphic xenobiotic enzymes. It could reasonably be expected that persons with increased propensity for PAH activation (*CYP1A1*) and decreased ability for detoxication of activated PAHs (*GSTM1*) would be at greater risk of PAH-related esophageal dysplasia. For *GSTM1* in the study presented here this hypothesis appears to be correct. For *CYP1A1* a mouse model shows that strains with higher *CYP1A1* enzyme levels have increased PAH-DNA adducts and are more sensitive to PAH induced tumorigenesis [32]. In this study, a similar relationship was not demonstrated for *CYP1A1*. This may be because the association of *CYP1A1\*2B* and elevated PAH metabolism remains conjectural [33]. In addition, even if this association was strong, redundancy in PAH metabolism may render a single polymorphism like *CYP1A1\*2B* ineffectual in regards to overall disease risk. Alternatively, it could be argued that a small, but insignificant effect, was observed (OR = 1.19) and a larger study population is needed to demonstrate the effect. In the case of *CYP2E1* and *GSTT1* a relationship with metabolic polymorphisms and nitrosamine or other environmental exposures and risk of dysplasia was hypothesized; however, none was revealed in the current study.

This study also demonstrates the capability of an esophageal cytologic sampler to provide DNA from the end organ of interest in evaluations of esophageal cancer. In the future, samples obtained by this method

may be useful for examining both genetic polymorphisms and biomarkers of local cumulative exposure, such as DNA adducts. Such studies may help to further examine the gene-environment interactions involved in the initiation and progression of this disease.

In summary, this study found that asymptomatic adults from the high risk region of Linxian, China who have a *GSTM1\*0* genotype appear to be associated with an increased risk of having histologic esophageal squamous dysplasia on screening endoscopy. This association suggests that exposure to environmental carcinogens that are detoxified by *GSTM1*, such as polycyclic aromatic hydrocarbons, may contribute to the etiology of squamous esophageal cancer in Linxian.

## Acknowledgements

The authors thank Wing Quan of the Laboratory of Cellular Carcinogenesis and Tumor Promotion, Division of Biological Sciences, National Cancer Institute, NIH, Bethesda, Maryland for his technical assistance.

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